Reference: *Biol. Bull.* **178:** 267–278. (June, 1990)

Efferent Innervation to *Limulus* Eyes *In Vivo* Phosphorylates a 122 kD Protein

SAMUEL C. EDWARDS*[,1], ANNE W. ANDREWS[1], GEORGE H. RENNINGER[2], ERIC M. WIEBE[1], AND BARBARA-ANNE BATTELLE[1]

[1]*The Whitney Laboratory and the Department of Neuroscience of the University of Florida, 9505 Ocean Shore Boulevard, St. Augustine, Florida 32086, and* [2]*Biophysics Interdepartmental Group, Department of Physics, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

Abstract. Efferent fibers innervate all of the eyes of the horseshoe crab, *Limulus polyphemus.* Driven by a circadian clock located in the central nervous system, the activity of the fibers at night is responsible for anatomical, biochemical, and physiological changes in the eyes, which increase their ability to detect and respond to light. We showed previously that octopamine, a putative efferent neurotransmitter, stimulates the phosphorylation of a 122 kD protein in *in vitro* preparations of both ventral and lateral eyes by means of a cAMP-dependent mechanism. We now report that phosphorylation of the 122 kD protein in the lateral eye is enhanced *in vivo:* (1) at night, in correlation with efferent nerve input activated by the circadian clock; and (2) during the day, in response to electrical stimulation of efferent axons. We show further that the 122 kD protein is enriched in, and may be restricted to, tissues that contain photoreceptors. We postulate that this protein is involved in the efferent-stimulated increase in retinal sensitivity.

Introduction

Retinal efferent neurons project to the eyes of many different vertebrate and invertebrate animals (See Evans *et al.,* 1983; Uchiyama, 1989), and in most species the function of this efferent input is unknown. The best understood of the retinal efferent systems is the one projecting to the eyes of the horseshoe crab, *Limulus polyphemus.* Efferent neurons innervate all of the eyes of *Limulus*—lateral, ventral, and median (Fahrenbach, 1971, 1981; Evans *et al.,* 1983). These neurons are, in turn, driven by a circadian clock located in the central nervous system (Barlow, 1983) such that they become active at night and are silent during the day (Barlow *et al.,* 1977). When the neurons are active, the anatomy, biochemistry, and electrophysiology of cells in the lateral eye change, leading to an increase in the ability of the eye to detect and respond to light (reviewed in Barlow, 1983).

Previous studies provide substantial evidence that the biogenic amine octopamine (OCT) is a neurotransmitter in the efferent neurons (Battelle *et al.,* 1982; Evans *et al.,* 1983; Battelle and Evans, 1984), and that this amine mimicks many of effects of efferent input to the eyes (Kass and Barlow, 1984; Kass and Renninger, 1988; Kass *et al.,* 1988; Pelletier *et al.,* 1984; Renninger *et al.,* 1989). Furthermore, many of these effects may be mediated by an OCT-stimulated increase in the intracellular second messenger, adenosine 3′,5′-monophosphate (cAMP). OCT increases the intracellular concentration of cAMP in lateral eye slices (Battelle and Wishart, unpub. obs.) and in the ventral eye (Kaupp *et al.,* 1982); and analogues of cAMP and agents that increase the level of intracellular cAMP—such as forskolin, a nonspecific adenylate cyclase stimulator (Seamon *et al.,* 1981)—mimic the physiological effects of efferent input or OCT *in situ* and in isolated tissues (Kass and Barlow, 1981, 1984; Kass and Renninger, 1988; Kass *et al.,* 1983, 1988; Pelletier *et al.,* 1984; O'Day and Lisman, 1985; Renninger *et al.,* 1988, 1989; Stieve, pers. comm.). Thus, we suspect that some of the effects of efferent innervation on

Received 3 December 1989; accepted 9 March 1990.

* Corresponding author and the author to whom reprint request should be sent. Present address: Department of Biology, University of South Florida 4202 Fowler Avenue, Tampa, FL 33620.

Abbreviations: cAMP PK, cAMP-dependent protein kinase; ERG, electroretinogram; OCT, octopamine; LON, lateral optic nerve; PAGE, polyacrylamide gel electrophoresis; SEM, standard error of the mean.

visual function may be mediated by an OCT-stimulated increase in cAMP.

Many of the effects of cAMP on cell function are thought to be mediated by the activation of cAMP-dependent protein kinase (cAMP PK) and the phosphorylation of specific substrate proteins (Nestler and Greengard, 1984). In a previous study we identified a 122 kD phosphoprotein, which, in isolated lateral eye slices and ventral eye photoreceptors, is phosphorylated in response to OCT via a cAMP-dependent mechanism (Edwards and Battelle, 1987). This led us to predict that, when efferent fibers become active in the animal, they release OCT into the eyes and stimulate a rise in intracellular cAMP. Cyclic AMP then activates cAMP PK and increases the phosphorylation of the 122 kD protein in retinal cells. To test this prediction we have used a back phosphorylation assay (Valtora *et al.*, 1986) to compare the level of phosphorylation of the 122 kD protein in lateral eyes deprived of efferent input and lateral eyes that received efferent input *in situ:* (1) at night, after efferent neurons were activated by the circadian clock in the central nervous system; and (2) during the day, in response to the electrical stimulation of the efferent axons.

In this back phosphorylation assay, proteins are fractionated by SDS polyacrylamide gel electrophoresis (PAGE), blotted onto nitrocellulose, and incubated in vitro with [γ-^{32}P] ATP and exogenous, purified, catalytic subunit of cAMP PK. The incorporation of $^{32}PO_4$ into specific proteins is then quantified by autoradiography and liquid scintillation spectroscopy. The amount of $^{32}PO_4$ that becomes associated with a protein is a measure of the number of cAMP PK substrate sites that are present in the dephosphorylated form. Thus, a previous increase in the phosphorylation of these sites by endogenous cAMP PK would be reflected as a *decrease* in the amount of radiolabeled phosphate that is incorporated into the protein in the back phosphorylation assay.

The results of our assays show that activation of the efferent axons *in situ,* during the night or during the day, stimulates the endogenous phosphorylation of the 122 kD protein. Furthermore, the tissue distribution of the 122 kD protein suggests that it is a prominent component of photoreceptor cells, and that it is enriched in, and may be restricted to, tissues that contain photoreceptors.

Materials and Methods

Animals

Adult, intermolt *Limulus polyphemus,* collected in the Indian River near Oak Hill, Florida, were maintained in running, natural seawater (15–18°C) on a 12 h light:12 h dark cycle. Animals were fed once a week and allowed to adapt to these conditions for at least two weeks before they were used.

In vitro *experiments*

Both lateral eyes from an animal were removed during the afternoon and placed into MOPS buffered *Limulus* saline (Warren and Pierce, 1982) containing 5 m*M* glucose. The corneas were removed, and small slices (approximately 1 × 2 mm), cut from the central region of the eyes, were placed into individual wells containing 1 ml of saline and incubated at room temperature for 1 h. They were then transferred to either fresh saline or saline containing 2 μM OCT (Sigma, St. Louis, Missouri) or 10 μM forskolin (Calbiochem, San Diego, California) and incubated for an additional 10 min. The tissues were homogenized in Laemmli (1970) SDS buffer, and the resulting homogenates were sonicated in a bath sonicator (Model W-225, Heat Systems-Ultrasonics Inc., Farmingdale, New York) and stored at 4°C pending analysis.

In vivo *experiments*

(1) Endogenous efferent activity. Each experiment was done using a single animal to eliminate animal to animal variation. One of the animal's eyes received endogenous efferent input, while input to the other eye was blocked by transecting the lateral optic nerve (LON) as described below.

An animal was attached to a rigid platform, and the LON of one eye was exposed—through a circular opening (approximately 2 cm in diameter) cut in the carapace just anterior to the eye—and transected. In two of the four experiments, the LON of the other eye was also exposed and served as a sham control; results with sham-operated eyes were the same as those in which the sham operation was not performed. After the opening was closed with gauze and beeswax, the animal was placed in the dark in an aquarium containing enough aerated, natural seawater to keep the book gills submerged. Visual sensitivity was monitored by recording the electroretinogram (ERG) as described by Horne and Renninger (1988). A microcomputer controlled the duration of the light stimulus (a green light-emitting diode, 100 ms), the interval between stimuli (10 min), and the recording of ERG activity.

Four hours after the onset of the animal's subjective night, as determined by an increase in the sensitivity of the eye receiving endogenous efferent innervation, both of its eyes were removed in the dark and immediately placed into liquid N_2. Then, under dim red light, each eye was quickly thawed by immersion in cold saline, the cornea was removed, and the eye was homogenized in SDS buffer with a glass-glass homogenizer. Each sample was sonicated, centrifuged for 5 min at 450 × *g* to remove the insoluble pigment granules, and the resulting supernatant was stored at 4°C until it was analyzed.

(2) Optic nerve stimulation. Early during an animal's

subjective day, it was fixed to a platform, as described above. Both LONs were exposed through circular openings cut in the carapace, and the LON to one eye was prepared for electrical stimulation (Barlow, 1983). Briefly, the LON was drawn into a small, saline-filled recording chamber that fit snugly in the opening in the carapace. The LON was dissected free of the surrounding blood vessel and cut. The cut end of the nerve that was attached to the eye was placed in a suction electrode filled with saline, and the lead of the electrode was connected through a stimulus isolation unit (Grass Model SIU5, Grass Instrument Co., Quincy, Massachusetts) to a pulse stimulator (Grass Model S88). The exposed LON of the other eye was left intact and served as a sham control. ERGs of both eyes were recorded, and the animal was maintained in the dark until the ERG activity stabilized (approximately 2 h). The LON was then electrically stimulated continually for 9 min with 15 or 30 volts DC (4 pulses per s, 2 ms pulse duration). The stimulus was turned off for 1 min while the ERG amplitude of the stimulated eye was monitored. This stimulus paradigm was repeated until the ERG amplitude of the stimulated eye reached a sustained, apparently maximal, level. Then both the stimulated and control eyes were removed from the animal and prepared for analysis, as described in the preceding section.

Back phosphorylation procedure

The level of phosphorylation of the 122 kD protein in *Limulus* visual tissue was examined by means of the back phosphorylation procedure developed by Valtorta *et al.* (1986) for proteins electrophoretically transferred and immobilized on nitrocellulose. Proteins from the lateral eyes were separated by SDS-PAGE (Laemmli, 1970) (7.5% acrylamide separating gel) and then electrophoretically transferred to a sheet of nitrocellulose (Towbin *et al.*, 1979). A prestained form of β-galactosidase (MW 116 kD; Sigma) was loaded into alternate lanes of the SDS gel to help locate the 122 kD protein on the nitrocellulose blot. Using ^{32}P-labeled 122 kD protein that had been prepared by treating lateral eye homogenates with [γ-^{32}P] ATP in the presence of 8-bromo cAMP (Edwards and Battelle, 1987), we determined that the transfer of 122 kD protein present in the gels to nitrocellulose was roughly quantitative and reproducible (coefficient of variation 15% for 5 experiments, data not shown).

Preliminary experiments showed that the ratio of the amount of 122 kD protein per mg total tissue protein varied from eye to eye; thus different volumes of each tissue sample were analyzed by the back phosphorylation procedure. Comparisons were then made between samples containing approximately the same amount of 122 kD protein as determined by Coomassie blue G250 staining of an identical gel, or by fast green staining of the protein on the nitrocellulose blot [0.1% (w/v) in 50% methanol and 10% acetic acid] following the assay.

The assay was usually performed only on the portion of the nitrocellulose blot containing the 122 kD protein, although in one experiment, shown in Figure 3, the level of phosphorylation of other proteins was examined. The blot was rinsed 3 times, for 5 min each, in 50 m*M* Tris and 200 m*M* NaCl at pH 7.4; it was then blocked by incubation for 1 h in the same solution containing, in addition, 0.4% (w/v) Ficol 400 and 0.1% Triton X100 (Valtorta *et al.*, 1986). The blot was then incubated for 1 h at 22°C, with continuous agitation, in a HEPES buffer, 50 m*M* (pH 7.4) containing 25 m*M* NaCl, 10 m*M* $MgCl_2$, 1 m*M* EGTA, 0.1 m*M* 2-mercaptoethanol, 0.1% Triton X100, 0.5 mg BSA, 76 n*M* Tris ATP, 15 μCi/ml [γ-^{32}P] ATP (NEN/Dupont, Wilmington, DE), and 1–5 units/ml of the catalytic subunit of cAMP PK (Sigma). The stock cAMP PK was routinely stored at 4°C in the solution described by Beavo *et al.* (1974). After the incubation, the blot was washed repeatedly, dried, and exposed with Kodak XAR X-ray film. The blotted proteins were then visualized using fast green, the 122 kD protein band was cut from the blot, and the radioactivity associated with it was determined by liquid scintillation spectroscopy.

The amount of labeled phosphate incorporated into the 122 kD protein from the unstimulated eye was expressed as 100%. The relative amount of *endogenous phosphorylation* of the 122 kD protein extracted from eyes receiving efferent nerve input was determined from the difference in the amount of labeled phosphate incorporated into the protein from efferent-stimulated and unstimulated eyes. For example, if the amount of $^{32}PO_4$ incorporated into the protein from the eye receiving efferent nerve input was 60% of that incorporated into the protein extracted from unstimulated eye, the remaining 40% was considered as being due to endogenous phosphorylation.

Validation of the back phosphorylation assay for the 122 kD protein in Limulus *tissue*

The *Limulus* LON contains a relatively large amount of the 122 kD protein that can be phosphorylated in homogenates by activation of endogenous cAMP PK (Edwards and Battelle, 1987; this study). The resulting $^{32}PO_4$ can subsequently be removed from the 122 kD protein by treating the tissue homogenates with alkaline phosphatase (unpub. results). To determine whether the back phosphorylation procedure would detect changes in the phosphorylation of the 122 kD protein, we measured the level of $^{32}PO_4$ incorporation into the blotted protein that had previously been incubated under phosphorylating

conditions, or incubated under phosphorylating conditions and subsequently stripped of phosphates with alkaline phosphatase. Specifically, aliquots of LON homogenates were incubated for 30 min at 30°C in media containing nonradioactively labeled ATP (30 μM) with, or without, 10 μM 8-bromo cAMP (Sigma) (Edwards and Battelle, 1987). At the end of the incubation period, several aliquots were treated with 2X SDS buffer and sonicated as described above. Other aliquots were mixed with an equal volume of 0.2 M Tris HCl (final pH 8.2) containing 2 mM phenylmethylsulfonyl fluoride, 2 mM $ZnCl_2$, 0.2 mM leupeptin, and 100 units/ml aprotinin, and then incubated for 2 h at 37°C with, or without, 81 units/ml calf intestinal alkaline phosphatase (type VII, Sigma) before the addition of SDS buffer. Samples containing the same amount of tissue protein were separated by SDS PAGE, blotted onto nitrocellulose, and the relative level of phosphorylation of the 122 kD protein for each treatment was determined using the back phosphorylation procedure.

Tissue distribution of the 122 kD protein

To determine the tissue distribution of the 122 kD protein in *Limulus*, samples from visual and nonvisual nervous tissues were homogenized in SDS buffer, sonicated and stored at 4°C until they were analyzed. The amount of protein in each sample was determined by a modified Lowry procedure (Peterson, 1977) using bovine serum albumin as the standard. Tissue samples containing an equal amount of protein were subjected to SDS PAGE, and proteins were visualized by silver stain (Heukeshoven and Dernick, 1985).

Results

Validation of the back phosphorylation using LON homogenates

Figure 1 shows that the 122 kD protein from the LON is a substrate for phosphorylation by purified catalytic subunit of cAMP PK and [γ-$^{32}PO_4$] ATP, even after it is separated from other tissue proteins by SDS-PAGE and electrophoretically transferred onto nitrocellulose. Prior treatment of the LON homogenate with 8-bromo cAMP resulted in a substantial reduction in the amount of radiolabeled phosphate ($^{32}PO_4$) associated with the 122 kD protein; subsequent incubation of the cAMP-treated homogenate with alkaline phosphatase returned the level of associated $^{32}PO_4$ to the untreated control level. The amount of 122 kD protein, as determined by fast green staining, was the same in all three samples.

Prolonged incubation (30 min) of the LON homogenate with 8-bromo cAMP did not produce a 100% reduction in the amount of $^{32}PO_4$ that subsequently became

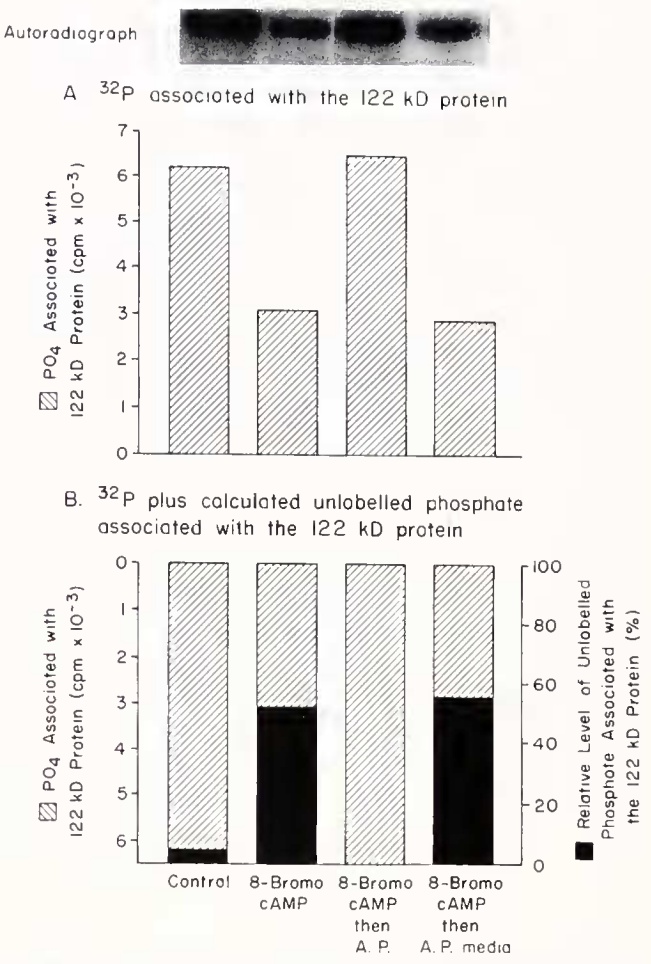

Figure 1. Back phosphorylation of homogenates of the *Limulus* LON demonstrates how the technique can be used to examine the level of cAMP-dependent phosphorylation of the 122 kD protein. Homogenates of LON were prepared and then incubated, as previously described (Edwards and Battelle, 1987), at 30°C in media containing ATP (30 μM) without (Control), or with, 10 μM 8-bromo cAMP. Some aliquots were then mixed with an equal volume of a 0.2 M Tris HCl buffered solution (A.P. media, final pH 8.2) and incubated for 2 h at 37°C with, or without, 81 units/ml calf intestinal alkaline phosphatase (A.P., type VII, Sigma). After all of the samples were solubilized, aliquots of each, containing an equal amount of total tissue protein (20 μg), were fractionated by SDS-PAGE and transferred onto nitrocellulose. The region of the blot containing the 122 kD protein was then subjected to the back phosphorylation procedure (See Methods). The association of $^{32}PO_4$ with the protein was first visualized with autoradiography (top of figure) and then quantified by liquid scintillation spectroscopy (A). The relative level of unlabeled phosphate associated with the protein (B) in each sample was calculated as the difference in the amount of phosphate in that sample compared to the alkaline phosphatase-treated sample. In the experiment shown, the phosphorylation of the 122 kD protein in aliquots incubated with ATP plus 8-bromo cAMP was 52.0% greater than that in aliquots incubated with ATP but without 8-bromo cAMP. In two similar experiments, 8 bromo cAMP stimulated a 23.9% and 28.4% increase in phosphorylation.

In another experiment, samples treated with SDS buffer immediately after homogenization were compared to ones treated with alkaline phosphatase; the amount of $^{32}PO_4$ associated with the 122 kD protein in these two cases was indistinguishable (data not shown). Thus the 122 kD protein seems not to be phosphorylated on cAMP-dependent sites in the intact LON.

associated with the 122 kD protein; the maximum reduction observed was 52% (Fig. 1), and the average percent reduction [± the one standard error of the mean (SEM)] for three separate experiments was 34.8 ± 12.3 (See the legend to Fig. 1). There are at least two possible explanations for this relatively small reduction in the incorporation of $^{32}PO_4$. (1) Sites on the protein normally phosphorylated by cAMP PK in tissue homogenates were not fully occupied by phosphate, even after the homogenate was exposed to 8-bromo cAMP and ATP for 30 min. (2) Treating the 122 kD protein with SDS and blotting it onto nitrocellulose exposes sites for phosphorylation that normally are not available in the protein's native configuration.

We draw two conclusions from these results. First, the reduced incorporation of $^{32}PO_4$ into the 122 kD protein of cAMP-treated homogenates reflects enhanced phosphorylation of the protein prior to the assay. That is, the amount of $^{32}PO_4$ incorporated into the protein on the blot is inversely related to the level of phosphorylation of the protein prior to the assay. Therefore, the back phosphorylation procedure can be used to assay the relative level of phosphorylation of the 122 kD protein. For example, in an experiment similar to that shown in Figure 1, we observed that the level of phosphorylation of the 122 kD protein in an alkaline phosphatase-treated LON homogenate was approximately the same as a sample in which SDS buffer was added immediately following homogenization (data not shown). We interpret this to mean that, at least during the day when all of these experiments were performed, the cAMP PK-dependent phosphorylation sites on the 122 kD protein in the LON are not phosphorylated *in vivo*.

Our second conclusion is that the maximum relative reduction in incorporation of $^{32}PO_4$ into the 122 kD protein that we may expect to observe, using this assay, is approximately 50%. We are assuming here that incubation *in vitro* for 30 min in the presence of 8-bromo cAMP and ATP stimulates the phosphorylation of nearly all of the sites available to cAMP PK in the native 122 kD protein.

OCT- and forskolin-stimulated phosphorylation of the 122 kD protein in slices of the lateral eye

With conventional *in vitro* phosphorylation procedures, we demonstrated an OCT-stimulated phosphorylation of the 122 kD protein in slices of the lateral eye in 3 of 8 (38%) experiments (Edwards and Battelle, 1987). The OCT-stimulated phosphorylation of the 122 kD protein in lateral eye slices is confirmed here using the back phosphorylation procedure (Fig. 2). Furthermore, the OCT-enhanced phosphorylation of the 122 kD protein in lateral eye slices was more reproducible using the back phosphorylation procedure (6 of 8, or 75% of the experiments). At least two factors may contribute to this increased reproducibility. (1) The 122 kD protein may have been extracted more efficiently by the homogenization procedure used in the present experiments (homogenization using a glass-glass homogenizer) as compared to the sonication procedure used previously. (2) Changes in the level of phosphorylation of the 122 kD protein may have been obscured in the conventional experiments due to high background radioactivity in the autoradiographs (See Figure 1a in Edwards and Battelle, 1987).

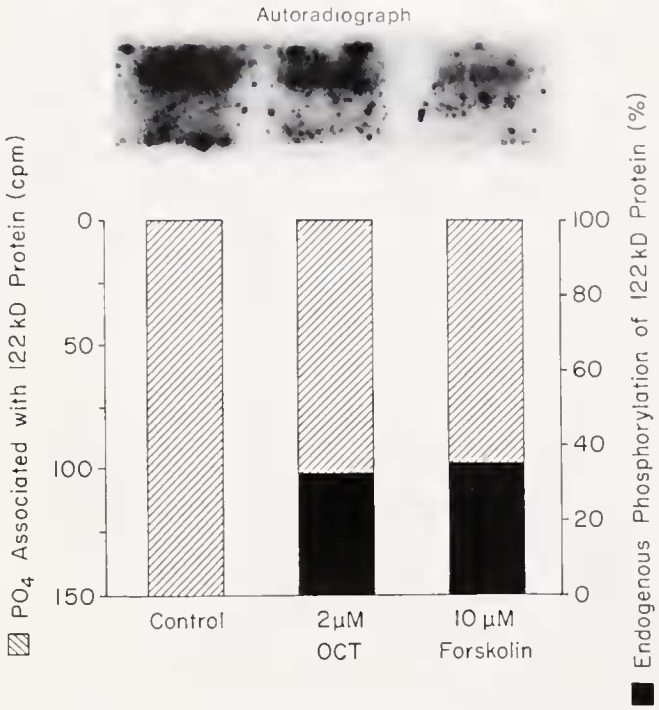

Figure 2. Octopamine- and forskolin-stimulated phosphorylation of the 122 kD protein in *Limulus* lateral eye slices *in vitro* as determined by the back phosphorylation procedure. Isolated tissues were incubated for 10 min in saline (Control), or saline containing 2 μM octopamine (OCT), or 10 μM forskolin (FOR) prior to analysis using the back phosphorylation procedure. This figure presents the results of one experiment. Shown are the autoradiograph, the amount of $^{32}PO_4$, and the calculated relative level of unlabelled PO_4 (endogenous phosphorylation) associated with the 122 kD protein in treated samples containing the same amount of 122 kD protein. The amount of protein as determined by fast green staining on the nitrocellulose blot. The results were similar in four replicates.

The 122 kD protein was also consistently (8 of 8 experiments) phosphorylated in lateral eye slices incubated with forskolin, a nonspecific adenylate cyclase stimulator (Fig. 2).

Changes in the level of 122 kD protein phosphorylation in vivo *in response to:*

(a) Endogenous efferent activity at night. To test our hypothesis that the 122 kD protein becomes phosphory-

lated at night in response to endogenous activation of the retinal efferent neurons, we compared the level of phosphorylation of the 122 kD protein in extracts of lateral eyes that had received endogenous efferent nerve input with those that had been deprived of this input by transection of the LON (Barlow, 1983). Because efferent activity and an increase in the sensitivity of the eye, as measured by an increase in ERG amplitude, are strongly correlated (Barlow, 1983), we monitored the ERG activity of the intact eye to determine when the efferent fibers became active.

In the experiment shown in Figure 3, the ERG amplitude began to increase above the daytime level at approximately 17:00 and continued to increase until it reached a maximum, sustained level 30 min to 1 h later (18:00). At 22:00, both eyes were quickly removed from the animal, processed for SDS-PAGE, and then analyzed for the relative level of endogenous phosphorylation of the 122 kD protein. In this experiment we measured a 34.7% increase in the level of endogenous phosphorylation of the 122 kD protein in the eye receiving efferent input compared to the control eye that had been deprived of efferent nerve activity (See Table IA; animal 1, assay 1). Three separate assays of material from the same animal gave a mean increase (± one SEM 4) of 36 ± 8.4%. Similar results were obtained with two other animals (Table IA).

Similar results were also obtained in an additional experiment in which the eyes of the animal were removed just before the onset of subjective day. In that experiment, the level of endogenous phosphorylation of the 122 kD protein in the eye receiving efferent input was 42% greater than that in the deprived eye. This implies that the level of endogenous phosphorylation of the 122 kD protein remains elevated in response to efferent activity throughout the subjective night.

(b) Optic nerve stimulation during the day. The efferent input to the lateral eye can be activated during the subjective day by electrically stimulating the LON; this also results in an increase in the sensitivity of the eye (Barlow 1983). Therefore, we examined whether electrical stimulation of efferent axons during the day resulted in enhanced phosphorylation of the 122 kD protein in the lateral eye.

The animals were prepared, and one lateral optic nerve was stimulated as described in Materials and Methods. The ERG amplitude began to increase within 10 to 30 min after the onset of stimulation, and continued to increase until it reached a maximum level approximately 40 to 50 min after the onset of the stimulation (Fig. 4). The ERG amplitude of the unstimulated, sham operated eye was unchanged. When the amplitude of the ERG of the stimulated eye reached a stable, maximum level, both the stimulated eye and the unstimulated eye were removed, processed for SDS-PAGE, and the relative level of 122 kD phosphorylation in the two eyes was determined. In the experiment shown in Figure 4, the level of endogenous phosphorylation of the 122 kD protein increased 64.7% in the eye that received electrically stimulated efferent input compared to the intact, daytime, unstimulated eye (See Table IB; animal 2, assay 1). The average increase (± one SEM) measured in four separate assays of material from the same animal was 42.1 ± 2.4%, and in similar experiments performed with 2 other animals, this increase in endogenous phosphorylation measured 47.2 ± 11.2% and 14.5 ± 4.0% (Table IB). Therefore, the increase in endogenous phosphorylation we measured in electrically stimulated eyes during the day was in the same range as what we observed with endogenous efferent input.

Distribution of the 122 kD protein in Limulus *nervous tissue*

The 122 kD protein appears to be restricted to those tissues in *Limulus* involved in visual processes (Fig. 5). It is quantitatively a major protein in the lateral eye, the ventral eye, the lateral and median optic nerves, and the lamina. It is usually observed in the median eye (4 of 6 animals), but its relative abundance in this tissue is variable. It is not a major protein constituent in the medulla, the central body region, or other portions of the brain, more posterior portions of the central nervous system, or the leg nerve. Furthermore, it was not detected in the cardiac ganglion, a tissue in which OCT receptors may also be present (Watson and Augustine, 1982; Groome and Watson, 1987).

Discussion

In this study we present strong evidence that efferent nerve activity stimulates the phosphorylation of a 122 kD protein in *Limulus* lateral eye. Efferent nerve activity could also modulate the amount of 122 kD protein in *Limulus* eyes, but because our assays were done on aliquots of LE extracts that contained the same amount of 122 kD protein, they reveal changes in the level of phosphorylation of the protein and not changes in its absolute amount.

The activity of the efferent nerves that project to *Limulus* eyes is driven by a circadian clock in the central nervous system, and efferent nerve activity causes an increase in the sensitivity of the lateral, ventral, and median eyes to light during the night (Barlow, 1983; Kass and Renninger, 1988). We show that enhanced phosphorylation of the 122 kD protein in lateral eyes *in vivo* correlates with the increased sensitivity of the lateral eye at night. We infer that the phosphorylation of this protein is regulated in a circadian manner, and we speculate

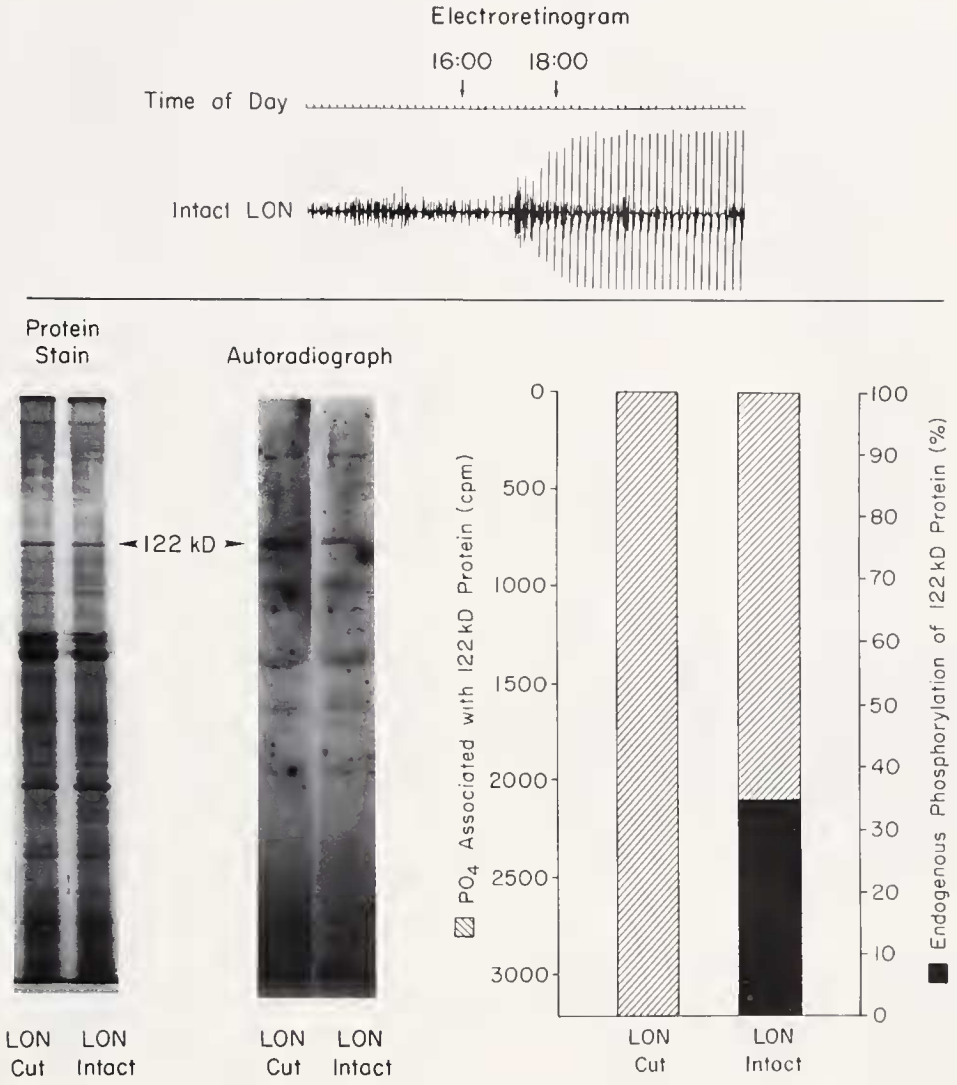

Figure 3. The 122 kD protein is phosphorylated *in vivo* in the *Limulus* lateral eye in response to activation of the efferent neurons by the circadian clock. This figure shows the results of a single assay (Table 1A; animal 1, assay 1). Early in the day, the LON was cut just anterior to one of the eyes to block efferent input to that eye. Input to the other eye was left intact. The animal was then placed into the dark in a standard apparatus for recording the ERG of the intact eye (Barlow, 1983). In the record shown, the ERG amplitudes were recorded every 10 min. Before 16:20, the small ERG amplitudes were partially obscured by voltage noise, probably arising from muscle contractions. After 16:20, the ERG amplitudes were clearly visible, and they began to increase after 17:10 as a result of efferent nerve activity. The ERG amplitudes (light sensitivity) stabilized at an elevated level by 19:00. At 22:00, both eyes were removed from the animal and immediately immersed in liquid N_2. Each eye was then briefly placed in ice cold saline so that the cornea could be removed, the tissue proteins were solubilized, and the amount of endogenous phosphorylation associated with the 122 kD protein from each eye was determined by the back phosphorylation procedure.

The lower portion of this figure shows (left to right): the pattern of proteins visualized by silver stain, (aliquots of solubilized preparations of both eyes contained about the same amount of 122 kD protein); an autoradiograph of a nitrocellulose blot containing larger (4×) aliquots of these samples, subjected to the back phosphorylation procedure; and the amount of $^{32}PO_4$ associated with the 122 kD protein from both eyes. The relative amount of *endogenous phosphorylation* in response to efferent stimulation was determined from the difference in the amount of labeled phosphate incorporated into the protein from efferent-stimulated and unstimulated eyes. The amount of labeled phosphate incorporated into the 122 kD protein from the unstimulated eye was expressed as 100%. The increase in endogenous phosphorylation observed in this assay was 34.7%; the mean increase (± one SEM) determined from three separate assays of material from the same animal was 36.0 ± 8.4%. Experiments with two other animals produced similar results; the results of all of these assays are presented in Table 1A.

Table I

Percent increase in the endogenous phosphorylation of the 122 kD protein following:

A. Endogenous efferent nerve activity[a]

	Assay number[b]			
Animal	1.	2.	3.	Mean ± SEM[c]
1.	34.7	31.4	32.8	33.0 ± 1.2
2.	28.9	26.4	52.6	36.0 ± 10.0
3.	36.1	48.7	44.7	43.2 ± 4.6

B. Electrically stimulated efferent nerve activity[d]

	Assay number[b]				
Animal	1.	2.	3.	4.	Mean ± SEM[c]
1.	40.3	41.3	38.7	41.1	42.1 ± 2.4
2.	64.7	33.8	43.2		47.2 ± 11.2
3	18.7	16.6	8.1		14.5 ± 4.0

[a] The experiment was performed on three separate animals exactly as described in the legend to Figure 3.

[b] For each animal studied, the relative level of phosphorylation of the 122 kD protein in homogenates of the unstimulated eye, and the eye receiving efferent input, were compared at least three separate times using the back phosphorylation procedure.

[c] Mean ± one SEM of three or four separate assays of the same set of homogenates.

[d] The experiment was performed on three separate animals exactly as described in the legend to Figure 4.

that the phosphorylation of this protein contributes to some aspects of the structural, physiological, or biochemical changes that occur in *Limulus* eyes in response to efferent nerve activity.

Direct correlation between efferent nerve activity and the phosphorylation of the 122 kD protein

The direct relationship between efferent nerve activity and the phosphorylation of the 122 kD protein is established by the combined results of the experiments done during the night and during the day. The observation that the 122 kD protein was relatively more phosphorylated at night in eyes receiving endogenous efferent nerve input, compared to eyes deprived of input, strongly suggests that enhanced phosphorylation of this protein is due to endogenous efferent nerve activity, but it does not eliminate the possibility that other factors, which may be present at night and not during the day, might also be required for the phosphorylation of the protein. However, results of the experiments in which we electrically stimulated the efferent axons during the day demonstrated that all factors necessary for the lateral eye to respond to efferent input with enhanced phosphorylation of the 122 kD protein are also present during the day.

Tissue distribution of the 122 kD protein

The 122 kD protein is a quantitatively major protein component of many of the tissues of the *Limulus* visual system. Its enrichment in the ventral eye, which consists predominately of photoreceptor cells (Clark *et al.*, 1969), argues that it is enriched in photoreceptor cells. Furthermore, the protein is very likely distributed throughout the photoreceptor cell, because it is found in both cell body- and axon-enriched portions of the ventral eye (Edwards and Battelle, 1987). But the protein may not be found exclusively in photoreceptor cells; much of the volume of the LON, which also contains a large amount of the 122 kD protein relative to other proteins, is composed of the large diameter axons of eccentric cells (Fahrenbach, 1971). It is also found in a high concentration in the first optic ganglia, or laminae, which contain the terminals of photoreceptor and eccentric cells from the lateral eye (Chamberlain and Barlow, 1980). The 122 kD protein may also be present in non-neuronal cells of the lateral eye.

The absence of a conspicuous 122 kD protein band in the medulla was surprising at first, because this tissue is innervated by cells from each of the eyes (Chamberlain and Barlow, 1980). This observation may be explained by a recent finding, which suggests that all photoreceptors from the lateral compound eye terminate in the lamina and do not innervate the medulla (Calman *et al.*, 1990). Thus, compared to the lamina, photoreceptor terminals in the medulla occupy a relatively low percentage of the total volume of the tissue.

The 122 kD protein may be distributed throughout the cells that contain it, but our evidence indicates that it is modified by phosphorylation in the somata of these cells and not in their axons. Previously we showed that activation of cAMP PK stimulated the phosphorylation of the 122 kD protein *in vitro* in portions of the ventral eye enriched in intact photoreceptor cell bodies (Edwards and Battelle, 1987); here we showed that the protein becomes phosphorylated in slices of the lateral eye *in vitro* and in the intact lateral eye *in vivo*. By contrast, in the LON, the 122 kD protein appears not to be a normal substrate of phosphorylation by cAMP PK. It becomes phosphorylated by activation of cAMP PK in broken cell preparations of the LON, but it is not phosphorylated in the LON *in vivo*, at least during the day (See Legend to Fig. 1), nor does it become phosphorylated in intact LON or axons of ventral photoreceptors in response to activation of cAMP PK (Edwards and Battelle, 1987). Consequently we believe that, in the optic nerves, the 122 kD substrate protein is physically separated from the cAMP PK.

The 122 kD protein is quantitatively the major substrate for cAMP PK in broken cell preparations of the

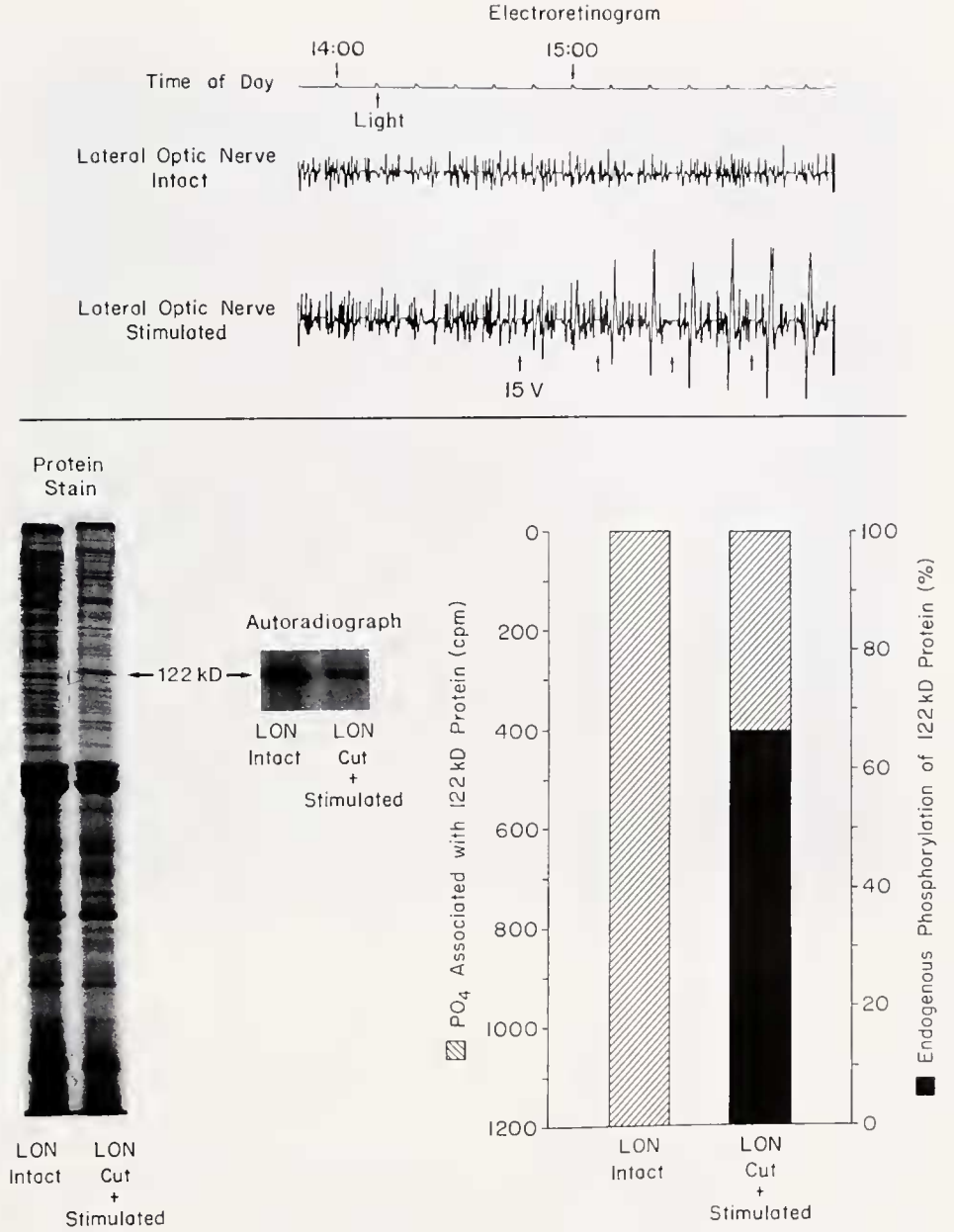

Figure 4. The 122 kD protein is phosphorylated *in vivo* in the lateral eye in response to electrical stimulation of the efferent axons. This figure shows the results of a single assay (Table 1B; animal 2, assay 1). Early in the day, the LON was cut just anterior to one of the eyes. After the animal was placed in the apparatus to record ERG activity, the cut end of the LON that remained with the eye was placed into a suction electrode. The animal was placed in the dark for 2 to 3 h, then the axons of the efferent neurons present in the LON were stimulated continuously with 15 V pulses (4 pps, 2 ms pulse duration) for 9 min (indicated by the arrow); the stimulation was then turned off for 1 min while the ERG amplitude was monitored. This sequence was repeated until the ERG amplitude reached a maximum, at which time both the stimulated and unstimulated eyes were removed and treated as described in Figure 3.

As in Figure 3, the ERG amplitudes of both eyes were initially obscurred by voltage noise. In the lower record, the ERG amplitudes began to increase after the second interval of electrical stimulation. The ERG amplitudes remained small in the upper (control) record.

The lower portion of this figure shows: the protein pattern visualized by silver stain (aliquots of solubilized preparations of both the stimulated and nonstimulated eyes contained about the same amount of 122 kD protein); an autoradiograph of a nitrocellulose blot containing larger (10×) aliquots of these samples, subjected to the back phosphorylation procedure; the amount of $^{32}PO_4$ associated with the 122 kD protein; and the calculated amount of endogenous phosphorylation of the protein in both samples. In the assay shown, we measured a 64.7% increase in endogenous phosphorylation of the 122 kD protein in the stimulated lateral eye of this animal; the average increase (± one SEM), determinated from separate assays of aliquots of the same extract, was 47.2 ± 11.2%. Experiments with two other animals produced similar results. The results of all assays are presented in Table 1B.

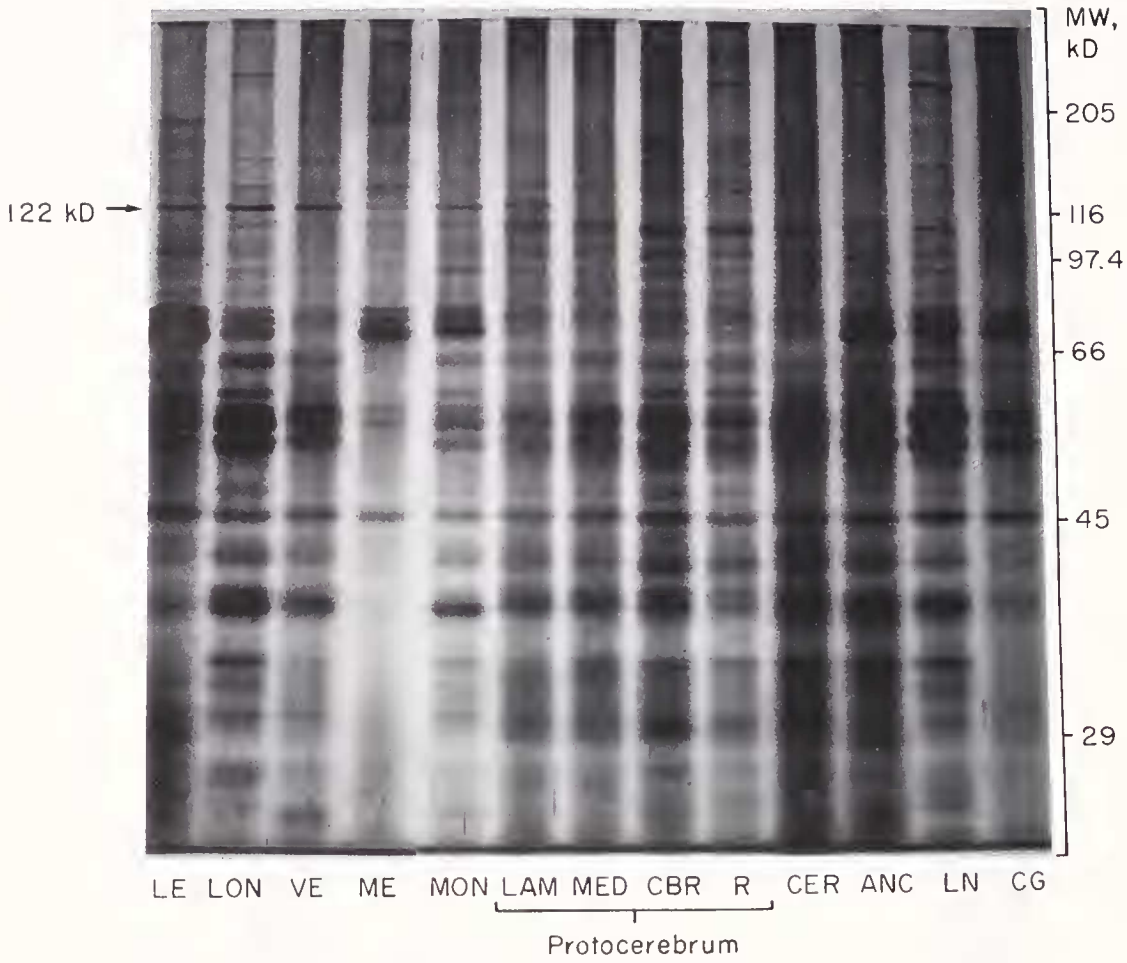

Figure 5. The 122 kD protein is a prominent protein component in *Limulus* eyes and optic nerves, and in the first optic ganglion. It does not appear prominently in other parts of the *Limulus* nervous system. Tissues were homogenized in 1× Laemmli (1970) SDS buffer. After the amount of protein in each sample was determined by a modified Lowry procedure (Peterson, 1977), aliquots containing 1 μg of total tissue protein for each sample were analyzed by SDS-PAGE and silver staining. Abbreviations: lateral eye (LE); lateral optic nerve (LON); ventral eye including P- and A-fractions (VE); median eye (ME); median optic nerve (MON); regions of the protocerebrum—first optic ganglion (lamina—LAM), medulla (MED), central body region (CBR), remaining portions (R); circumesophageal ring (CER); abdominal nerve cord (ANC); peripheral leg nerve (PLN); cardiac ganglion (CG).

lamina as well (data not shown). Because we have not yet examined whether it is phosphorylated in the intact tissue in response to agents that increase intracellular cAMP, the significance of these results are presently unknown.

A model for the regulation of visual function by efferent innervation

Our current model for how efferent nerve activity modulates the function of *Limulus* eyes is presented in Figure 6. The results described in the present study provide support for some aspects of this model.

The circadian nature of the efferent nerve input to *Limulus* eyes is well established (reviewed in Barlow, 1983), and there is convincing evidence that OCT is a neurotransmitter in the efferent axons. OCT is synthesized and stored in the efferent axons that project to the ventral and lateral eyes (Battelle *et al.*, 1982; Evans *et al.*, 1983), and it is released from these axons in a Ca^{2+}-dependent manner *in vitro* in response to veratridine (Battelle and Evans, 1986) or depolarization with high extracellular potassium (Battelle and Evans, 1984). OCT mimics many of the physiological effects of endogenous efferent innervation when applied to the lateral eye in situ (Kass and Barlow, 1984), or to *in vitro* preparations of either the lateral (Kass *et al.*, 1988; Renninger *et al.*, 1989), or ventral eye (Kass and Renninger, 1988). Clo-

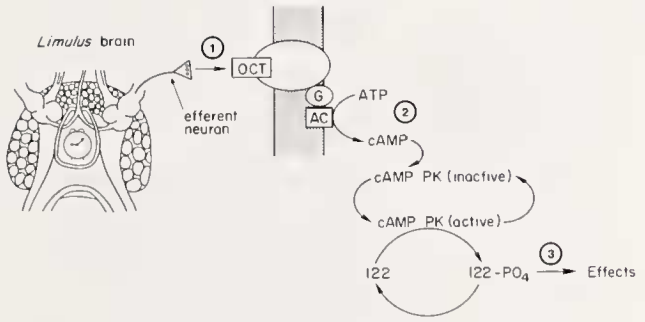

Figure 6. A model for the regulation of visual function by efferent innervation. (1) At night, a circadian clock in the central nervous system activates the efferent fibers innervating *Limulus* eyes, causing the release of the efferent neurotransmitter octopamine (OCT). (2) The interaction of OCT with OCT-specific receptors on the photoreceptors, and perhaps other cell types in the lateral eye, increases the activity of adenylate cyclase, thereby elevating intracellular cyclic AMP levels. (3) This results in the activation of cyclic AMP-dependent protein kinase which phosphorylates the 122 kD protein. We propose that this phosphoprotein is involved in the anatomical, biochemical, and physiological processes responsible for increasing the sensitivity of the eye.

zapine, an OCT-receptor blocker (Dougan and Wade, 1978; Evans, 1981), blocks both the physiological effects of exogenously added OCT and those generated by endogenous efferent activity when it is applied to the lateral eye *in situ* (Kass and Barlow, 1984). Direct evidence for the release of OCT from efferent fibers in response to electrical stimulation or endogenous activation by the circadian clock *in vivo* (Fig. 6, #1) is lacking, but as described below, the results of this study are consistent with this idea.

In the Introduction, we describe the results of many studies that suggest that many of the physiological effects of efferent activity in *Limulus* eyes are mimicked by cAMP. Thus, it is predicted that the release of neurotransmitter from efferent terminals will stimulate an increase in cAMP in *Limulus* eyes (Fig. 6, #2). Here we showed that efferent nerve input enhanced the phosphorylation of a major substrate for cAMP PK, the 122 kD protein (Edwards and Battelle, 1987; this study), at sites specific for purified cAMP PK. Because the 122 kD protein is not a substrate for Ca^{+2}/calmodulin protein kinase (Weibe *et al.*, 1989) or protein kinase C (Weibe, Calman, and Battelle, unpub. obs.), our results provide strong indirect evidence that efferent nerve input increases the intracellular concentration of cAMP in the lateral eye.

OCT, acting apparently through an OCT-specific receptor, stimulates a rise in intracellular cAMP in preparations of ventral and lateral eyes *in vitro* (Kaupp *et al.*, 1982; Battelle and Wishart, unpub. obs.). Thus, our current results are also consistent with the idea that OCT is released in response to efferent fiber activity *in vivo* (Fig. 6, #1). However, we cannot exclude the possibility that the 122 kD protein is phosphorylated in response to another, unidentified neurotransmitter that is released from efferent terminals and acts at a receptor coupled to adenylate cyclase.

Several observations lead us to predict that the 122 kD protein is involved in some aspect of the efferent-stimulated changes in retinal function (Fig. 6, #3). Its phosphorylation is stimulated by efferent innervation, and correlates with efferent stimulated changes in visual sensitivity. It is a major substrate for cAMP PK in *Limulus* eyes, and cAMP is believed to mediate many of the effects of efferent innervation on retinal function. Further analysis of the role of this protein in efferent-stimulated changes in visual function requires a detailed characterization of the protein and its cellular distribution.

Is the 122 kD protein the only protein phosphorylated in response to efferent innervation?

The 122 kD protein was the only detectable substrate for OCT stimulated phosphorylation in intact retinal cells *in vitro* (Edwards and Battelle, 1987), and in the experiment shown in Figure 3 of this study, the 122 kD protein was the only one that showed a detectable change in phosphorylation that correlates with efferent input *in vivo*. However, we wish to emphasize that, in the present study, conditions were optimized specifically to examine changes in the level of phosphorylation of the 122 kD protein, and changes in the phosphorylation of quantitatively more minor protein components may have been missed. Studies with broken cell preparations revealed other potential substrates for cAMP-dependent phosphorylation (Edwards and Battelle, 1987), but it is unclear whether these are relevant substrates in intact cells.

Because the effects of efferent nerve activity, OCT, and cAMP on retinal function in *Limulus* are many and diverse, the 122 kD protein is unlikely to be the only protein that becomes modified. But the 122 kD protein clearly is a major protein substrate in *Limulus* eyes, and it is phosphorylated, and presumably regulated, by cAMP-, OCT-, and efferent innervation.

Acknowledgments

We thank Lynn Milstead and James Netherton for assisting in producing the figures. This work was supported by basic research grants from the National Science Foundation (BNS 86-07660 and BNS 89-09052 to B-A.B.), and undergraduate research training grants from the National Science Foundation (BBS 87-12402) and the Grass Foundation (to E.M.W.).

Contributions of the Authors

S. C. Edwards was principally responsible for designing, conducting, interpreting, and describing this

study; A. W. Andrews performed the back phosphorylation assays; G. H. Renninger provided expertise critical to the performance of the electrophysiological assays; E. M. Wiebe set up the electronics required for monitoring retinal sensitivity and examined the distribution of the 122 kD protein. B-A. Battelle oversaw all aspects of the study, was heavily involved in its design and the interpretation of the data, and, together with G. H. Renninger, contributed significantly to the generation of the manuscript.

Literature Cited

Barlow, R. B., Jr. 1983. Circadian rhythms in the *Limulus* visual system. *J. Neurosci.* **3:** 856–870.

Barlow, R. B., Jr., S. J. Bolanowski Jr., and L. M. Brachman. 1977. Efferent optic nerve fibers mediate circadian rhythms in the *Limulus* eye. *Science* **197:** 86–89.

Battelle, B-A., and J. A. Evans. 1984. Octopamine release from centrifugal fibers of the *Limulus* peripheral visual system. *J. Neurochem.* **42:** 71–79.

Battelle, B-A., and J. A. Evans. 1986. Veratridine-stimulated release of amine conjugates from centrifugal fibers in the *Limulus* peripheral visual system. *J. Neurochem.* **46:** 1464–1472.

Battelle, B-A., J. A. Evans, and S. C. Chamberlain. 1982. Efferent fibers to *Limulus* eyes synthesize and release octopamine. *Science* **216:** 1250–1252.

Beavo, J. A., P. J. Bechtel, and E. G. Krebs. 1974. Preparation of homogeneous cAMP-dependent protein kinase(s) and its subunits from rabbit skeletal muscle. *Meth. Enzymol.* **38:** 299–308.

Calman, B. G., M. A. Lauerman, A. C. Wishart, M. Schmidt, and B-A. Battelle. 1990. A monoclonal antibody to photoreceptor cells in *Limulus*. *Invest. Ophthalmol. Vis. Sci. (suppl.)*: **31:** 286.

Chamberlain, S. C., and R. B. Barlow Jr. 1980. Neuroanatomy of the visual afferents in the horseshoe crab (*Limulus polyphemus*). *J. Comp. Neurol.* **192:** 387–400.

Clark, A. W., R. Millecchia, and A. Mauro. 1969. The ventral photoreceptor cells of *Limulus* I. The microanatomy. *J. Gen. Physiol.* **54:** 289–309.

Dougan, D. F. H., and D. N. Wade. 1978. Octopamine receptors and their structural specificity. *Clin. Exp. Pharmacol. Physiol.* **5:** 341–349.

Edwards, S. C., and B-A. Battelle. 1987. Octopamine- and cAMP-stimulated phosphorylation of a protein in *Limulus* ventral and lateral eyes. *J. Neurosci.* **7:** 2811–2820.

Evans, J. A., S. C. Chamberlain, and B-A. Battelle. 1983. Autoradiographic localization of newly-synthesized octopamine to retinal efferents in the *Limulus* visual system. *J. Comp. Neurol.* **219:** 369–383.

Evans, P. D. 1981. Multiple receptor types for octopamine in the locust. *J. Physiol. (Lond.)* **318:** 99–122.

Fahrenbach, W. H. 1971. The morphology of the *Limulus* visual system. IV. The lateral optic nerve. *Z. Zellforsch. Microsk. Anat.* **114:** 532–545.

Fahrenbach, W. H. 1981. The morphology of the *Limulus* visual system. VII. Innervation of photoreceptors by neurosecretory efferents. *Cell Tissue Res.* **216:** 655–659.

Groome, J. R., and W. H. Watson III. 1987. Mechanism for amine modulation of the neurogenic *Limulus* heart: evidence for involvement of cAMP. *J. Neurobiol.* **18:** 417–431.

Heukeshoven, J., and R. Dernick. 1985. Simplified method of silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* **6:** 103–112.

Horne, J. A., and G. H. Renninger. 1988. Circadian photoreceptor organs in *Limulus*. I. Ventral, median, and lateral eyes. *J. Comp. Physiol. A* **162:** 127–132.

Kass, L., and R. B. Barlow Jr. 1981. Pharmacological agents partially reproduce the effects of a circadian clock on *Limulus* lateral eye. *Biol. Bull.* **161:** 348.

Kass, L., and R. B. Barlow, R. B. Jr. 1984. Efferent neurotransmission of circadian rhythms in *Limulus* lateral eye. I. Octopamine-induced increases in retinal sensitivity. *J. Neurosci.* **4:** 908–917.

Kass, L., J. L. Pelletier, G. H. Renninger, and R. B. Barlow Jr. 1983. cAMP: a possible intracellular transmitter of circadian rhythms in *Limulus* photoreceptors. *Biol. Bull.* **164:** 378.

Kass, L., J. L. Pelletier, G. H. Renninger, and R. B. Barlow Jr. 1988. Efferent neurotransmission of circadian rhythms in *Limulus* lateral eye. II. Intracellular recordings in vitro. *J. Comp. Physiol. A* **164:** 95–105.

Kass, L., and G. H. Renninger. 1988. Circadian change in function of *Limulus* ventral photoreceptors. *Vis. Neurosci.* **1:** 3–11.

Kaupp, U. B., C. C. Malbon, B-A. Battelle, and J. E. Brown. 1982. Octopamine stimulated rise in cAMP in *Limulus* ventral photoreceptors. *Vis. Res.* **22:** 1503–1506.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of the phage T4. *Nature* **277:** 680–685.

Nestler, E. J., and P. Greengard. 1984. *Protein Phosphorylation in the Nervous system.* New York: Wiley.

O'Day, P. M., and J. E. Lisman. 1985. Octopamine enhances dark adaptation in *Limulus* ventral photoreceptors. *J. Neurosci.* **5:** 1490–1496.

Pelletier, J. L., L. Kass, G. H. Renninger, and R. B. Barlow Jr. 1984. cAMP and octopamine partially mimic a circadian clock's effect on *Limulus* photoreceptors. *Invest. Ophthalmol. Vis. Sci.* (Suppl.) **26:** 378.

Peterson, G. L. 1977. A simplification of the protein assay method of Lowry *et al.* which is more applicable. *Anal. Biochem.* **83:** 346–356.

Renninger, G. H., L. Kass, J. L. Pelletier, and R. Schimmel. 1988. The eccentric cell of the *Limulus* lateral eye: encoder of circadian changes in visual responses. *J. Comp. Physiol. A* **163:** 259–270.

Renninger, G. H., R. Schimmel, and C. A. Farrell. 1989. Octopamine modulates photoreceptor function in the *Limulus* lateral eye. *Vis. Neurosci.* **3:** 83–94.

Seamon, K. B., W. Padnett, and J. W. Daly. 1981. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Nat. Acad. Sci. U.S.A.* **78:** 3363–3367.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Nat. Acad. Sci. U.S.A.* **74:** 4350–4354.

Uchiyama, H. 1989. Centrifugal pathways to the retina: influence of the optic tectum. *Vis. Neurosci.* **3:** 183–206.

Valtorta, F., W. Schiebler, R. Jahn, B. Ceccarelli, and P. Greengard. 1986. A solid-phase assay for the phosphorylation of proteins blotted on nitrocellulose membrane filters. *Anal. Biochem.* **158:** 130–137.

Warren, M. K., and S. K. Pierce. 1982. Two cell volume regulatory systems in the *Limulus* myocardium: an interaction of ions and quaternary ammonium compounds. *Biol. Bull.* **163:** 504–516.

Watson, W. H., and C. J. Augustine. 1982. Peptide and amine modulation of the *Limulus* heart: a simple nervous network and its target tissue. *Peptides*, **3:** 485–492.

Wiebe, E. M., A. C. Wishart, S. C. Edwards, and B-A. Battelle. 1989. Ca^{++}/calmodulin-stimulated phosphorylation of photoreceptor proteins in *Limulus*. *Vis. Neurosci.* **3:** 107–117.